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Metabolic Fingerprinting of Three Malaysian Ginger (*Zingiber officinale* Roscoe) Using Gas Chromatography-Mass Spectrometry

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Abstract: Problem statement: There is a chemical variation among different ginger cultivars detected by previous studies including the volatile and non-volatile oleo-resins. In this study we try to determine whether these variations are related to a genetic factor rather than environmental or intrinsic factors. **Approach:** A comprehensive metabolic fingerprinting from the leaves of three micro-propagated ginger cultivars Bukit Tinggi, Tanjung Sepat and Sabah was performed using a Gas Chromatography-Mass Spectrometry (GC-MS). Constituents of the ginger leaves were first extracted and then fractionated into methanolic and chloroform. The samples were subsequently methoximated and silylated prior to GC-MS analysis. **Results:** By applying this technique, we detected more than 300 compounds (polar and non-polar) in total originated from each ginger cultivar. Based on the GC-MS fragmentation, three different classes of metabolites were detected from the ginger cultivars, namely amino acids, carbohydrates and organic acids. A qualitative variation on the type of ginger metabolites was observed, albeit no marked different found in the level of the metabolites. **Conclusion:** Apparently the chemical variations among the three ginger cultivars were due to genetic effects since almost all other environmental and intrinsic factors were eliminated.

Key words: Ginger, metabolic fingerprinting, gas chromatography-mass spectrometry, derivatisation

INTRODUCTION

Ginger (*Zingiber officinale* Roscoe) is an important tropical horticultural plant, values all over the world as a spice and for its medicinal properties. Ginger belongs to the family *Zingiberaceae*, which contains about 1300 species in 50 genera, along with four other families is placed in the order *Zingiberales* which belong to class *Monocotyledones* (Berg, 1997). The plant is completely sterile (produce no seed) and only propagated by rhizomes (Berg, 1997; Rout *et al.*, 1998). Members of the family have distributions in the tropics of South and South-Eastern Asia specially Indo-Malaysia (Simpson, 2006; Awang, 1992) and cultivated throughout the tropical and sub-tropical region, was among the first vegetative cultivated plants. The Chinese records showed ginger was cultivated in the Malacca region (Malaysia) in 1416 (Weiss, 2002). The Spanish introduced this spice into the new world, where it grew so successfully, that by 1547 (Levetin and McMahon, 2006). The underground rhizomes are the medicinally and culinary useful part of the plant (Syamkumar *et al.*, 2003).

The chemistry of ginger is well documented with the respect to the oleoresin and volatile oil (Barnes *et al.*, 2002; Sweetman, 2007). More than 400 chemicals have been identified in ginger rhizome (Garner-Wizard *et al.*, 2006). Geography, age of rhizome at harvest and extraction methods determine the relative proportions of chemicals. Chemical constituents of ginger classified to volatile oils which constitutes (1-3%) mainly of zingiberene (Robbers *et al.*, 1996; Fnimh, 2001) non-volatile pungent compounds oleo-resin constitute (4-7.5%) mainly gingerols and other constituents with more than 50% of starch (Robbers *et al.*, 1996), many fats, waxes, carbohydrates, vitamins and minerals. A difference in the type and level of major chemical constituents was detected among ginger rhizomes collected from different plantation regions (Ravindran and Babu, 2004). In the present study, we carry out the GC-MS based metabolic fingerprinting to examine whether the chemical variation are due to environmental factors or due to genetic factor. The chemical profiles of three micro-propagated ginger explants, namely Bukit Tinggi; Tanjung Sepat and Sabah cultivars using a metabolomic approach were detected. Ma and Gang (2006) reported the suitability

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of using tissue cultured ginger plant for detection of chemical variation. Ma and Gang shown that there are no significant differences existed between conventional greenhouses grown versus *in vitro* propagation-derived ginger plants.

Metabolic fingerprinting: Metabolic fingerprinting appears to be the easiest approach to metabolome analysis which utilizes all detector readings for numerical analysis to unambiguously identify the specific metabolites represented in these experiments. In addition, metabolic fingerprints are applied for metabolic pattern comparison aimed at the discovery of experimental conditions which result in similar or identical metabolic responses. This approach is used in gene function analysis and has the potential to group genes with known function and orphan genes of unknown or hypothetical function into classes of similar or identical metabolic function. This type of metabolic pattern analysis appears to be especially promising when gene modifications result in 'silent' phenotypes (changing of the metabolic state in organisms, which do not show obvious visual or morphological traits) (Steinhauser and Kopka, 2007).

Measuring large numbers of compounds was developed within the last few years (Last *et al.*, 2007). Hyphenated techniques that couple chromatography to mass spectrometry offer the greatest confidence in sample identification and quantification (Fiehn *et al.*, 2000; Dunn, 2008). Since gas chromatography is only suitable for thermally stable non-polar compounds, while we want to do a comprehensive detection (as possible) of all metabolites, so that a chemical derivatisation is needed. Derivatisation is a chemical reaction of a sample that yields a product that is more volatile and stable and that has improved gas chromatography behavior over the original substances (Kitson *et al.*, 1996). In most cases, derivatisation is performed to convert polar N-H, O-H and S-H groups into thermally stable, non-polar groups (Gullberg, 2005; Hübschmann, 2001). Metabolic fingerprinting, as one approach of metabolomic, is a comprehensive and high-throughput analysis of crude samples or sample extracts with minimal requirements for sample preparation (Dunn *et al.*, 2005). Metabolic fingerprinting is suitable to use for sample classification (Halket *et al.*, 2005) or sample screening which utilize all detector readings for numerical analysis (Fiehn, 2002). Metabolic fingerprinting analysis appears to be especially promising when gene modifications result in 'silent' phenotypes or silent mutation. Silent phenotypes are better defined as changes of the metabolic state in organisms, which do not show

obvious visual or morphological traits (Steinhauser and Kopka, 2007).

MATERIALS AND METHODS

In present study, we carried out a metabolic fingerprinting analysis for the leaves of micro-propagated ginger cultivars from Bukit Tinggi, Tanjung Sepat and Sabah in an attempt to detect chemical variations present among three ginger cultivars. The degree of correlation present among the ginger cultivars will be examined based on direct comparison of the ginger metabolites.

Plant materials: Nine-month old of ginger rhizomes cv. Bukit Tinggi, Tanjung Sepat and Sabah were obtained from there original plantation regions in Malaysia. A sterile young bud of ginger is used for initiating a stock culture of ginger plantlets grown in Murashige and Skoog (MS) media containing 3% (w/v) sucrose; 2 mg L⁻¹ 1-naphtalen acetic acid in combination with 2 mg L⁻¹ kinetin as growth hormones and agar 0.08% (w/v). Ginger explants were sub-cultured monthly to eliminate any carryover affects of prior growth conditions (from the original source) on the chemical composition and to ensure that uniform growth conditions were applied equally to all plants. Three month old ginger explants were used in this experiment (Fig. 1).

Chemicals: Chloroform, methanol absolute, n-heptan (Fisher Scientific), sodium chloride, sucrose, sulphuric acid, sodium bicarbonate, anhydrous sodium sulphate, (Merck), methyl nonadecanoate, methoxyamine hydrochloride (O-methyl hydroxylamine HCl), N-methyl-N-(trimethyl-silyl) trifluoroacetamide (MSTFA) (Sigma-Aldrich) and pyridine (AnalaR).



Fig. 1: Three months old ginger explants



Fig. 2: Picture of the GC-MS used in the study (Agilent Technologies 6890N network GC system)

Instruments: Mortar and pestle, Centrifuge (Rotofix 32, hettich Zentrifugen, Germany), Incubator shaker (INNOVA 4000, M1192, NEW BRUNSWICK SCIENTIFIC), Centrifuge evaporator (VR- maxi st. a.-1, Heto vacuum centrifuge, Heto-Holten A/s, Denmark), Incubator (precision incubator, BE 400, Memmert) and GC-MS system (Agilent Technologies 6890N network GC system) (Fig. 2).

Sample preparation: Sample preparation was done according to Jiang *et al.* (2006) method with a slight modification. For each ginger cultivar, three samples of explants (3 months old) were harvested; the leaves were cut and immediately immersed in to liquid nitrogen to stop any enzyme activity that may lead to change of chemical constituents. Subsequently, ginger leaves were grinded to fine powder while kept frozen under liquid nitrogen and homogenized. Then the plant samples were stored at -80°C freezer until further use (within 3 weeks).

Extraction of polar and non-polar metabolites: The extraction protocol was carried out according to Shepherd *et al.* (2007) with some modifications. Briefly, 500 mg of powdered frozen ginger leaves were weighed and immediately transferred to a glass culture tube (125×16 mm) containing 3 mL methanol. A non-polar internal standard 100 μL methanolic methyl nonadecanoate (0.2 mg mL^{-1}) and 100 μL sucrose (2.5 mg mL^{-1}) as a polar internal standard were added; shake vigorously on an incubator shaker for 30 min at 60°C . Distilled water (360 μL) was added and the sample was shaken for 30 min at 60°C , prior to addition of 6 mL of chloroform. The resulted mixture was further shaken for 60 min at 60°C . One and half milliliter distilled water was added and mixed

vigorously by hand and separated by centrifugation at a speed of 4000 rpm for 3 min. A polar methanolic fraction (upper layer) was separated from a non-polar chloroform fraction (lower layer) by pipette and transferred to 5 mL glass vial. Both fractions were either directly processing or alternatively were stored at -20°C until further use. Methanolic fractions could be stored immediately whereas chloroform fractions were first evaporated to dryness under nitrogen gas.

Derivatisation of methanolic fraction: Derivatisation of methanolic fraction was carried out according to Shepherd *et al.* (2007) with a slight modification. One milliliter of methanolic fraction was transferred into eppendorf tube and evaporated to dryness using a centrifuge evaporator. Subsequently, 40 μL of 20 mg mL^{-1} methoxyamine hydrochloride in anhydrous pyridine was added to the dry fraction and then the sample was incubated at 30°C for 90 min with continuous shaking. MSTFA (40 μL) was added and incubated at 37°C for 60 min. The derivatized sample (40 μL size) was diluted with 60 μL n-heptane and 1 μL sample was injected to GC-MS.

Derivatisation of chloroform fraction: For chloroform fraction, the protocol was carried out according to Shepherd *et al.* (2007) with a slight modification. Whole fraction was evaporated to dryness under dry pure nitrogen gas. Two milliliter of methanolic sulphuric acid (1% v/v) was added and incubated overnight at 50°C for trans-esterification. Five milliliters of sodium chloride (5% w/v) and 3 mL chloroform were added to the sample; the mixture was shaken and left to separate into two layers. The upper aqueous layer was discarded. To the lower chloroform layer add 3 mL of sodium bicarbonate (2% w/v), shake and leave to separate. The lower chloroform layer was removed, dried over anhydrous sodium sulphate and then evaporated to dryness using nitrogen gas. The dry fraction was solubilised in 50 μL chloroform and 10 μL pyridine. Silylation (derivatisation) was achieved with 80 μL MSTFA at 37°C for 60 min, after that, 40 μL of derivatized sample was taken, mixed with 60 μL n-Heptan and 1 μL sample was injected to GC-MS for analysis.

GC-MS conditions: The following operating parameters were used: capillary GC column HP-5MS 5% phenyl methyl siloxane (30×0.25 mm i.d. ×0.25 mm film thickness), a carrier gas Helium (flow rate 1.2 mL min^{-1}) and a split-less injection mode. Injector temperature is 250°C , Oven temperature will be set initially at 50°C , and then will be raised to 250°C at a

10°C min⁻¹ rate till the end of analysis. The eluted analytes detected using (5973 network) mass selective detector and Electron Impact ionization (EID) will be carried out at 70 eV. Data were acquired using Enhanced ChemStation G1701CA version C00.0021, Agilent technology. The identity of compounds will be checked using the Wiley7n.1 spectra data base.

RESULTS

The main criteria for selection of suitable ions for an identification of compound should has a high peak area (>0.05%) and should be unique and/or be well resolved from other ions with the same mass to charge ratio (m/z) in the defined time window. Identification of the compounds indicated by the library search program as being more than 80% and viewed as being likely hits (Jiang *et al.*, 2006). Compounds were identified by analysis of standards, comparison with wiley7n.1 database library. Spectra for each eluting compound were then compared manually to the data box and standard spectrum (NIST Chemistry WebBook) for the best hit to determine if the molecular ion peaks and the fragmentation patterns did in fact match. We found in total about 314-385 metabolites consisted of both polar and non polar compounds (Table 1) which was detected from the GC-MS metabolic fingerprinting of the leaves of three micro-propagated Bukit Tinggi, Tanjung Sepat and Sabah ginger cultivars.

Approximately 30% of the total number of peaks could be identified both in methanolic and chloroform fractions of Bukit Tinggi ginger cultivar, whereas, only about 24 and 25.7% of the total peaks were identified for Tanjung Sepat and Sabah ginger cultivars respectively. Furthermore, based on the GC-MS fragmentation, three different classes of metabolites were detected from the ginger cultivars, namely amino acids, carbohydrates and some organic acids.

The GC-MS chromatogram of the methanolic fraction (Fig. 3-5) and the chloroform fraction (Fig. 6-8) of the three samples of ginger leaves extract showed qualitatively apparent differences in the type of metabolites (Table 2 and 3). The polar fraction of the BT ginger cultivar contained a remarkably high level of sugars compared to two other cultivars at the retention time window around 33-42 min. The TS ginger cultivar showed a relatively high content of amino acids rather than the SB and TS cultivars at the retention time windows from 19-29 min.

Table 1: Number of metabolites detected in methanolic and chloroform fraction of different ginger cultivars

Ginger cultivar	Metabolites detected in methanolic fraction	Metabolites detected in chloroform fraction	Total No. of metabolites
Bukit Tinggi	207	178	385
Tanjung Sepat	174	141	315
Sabah	169	145	314

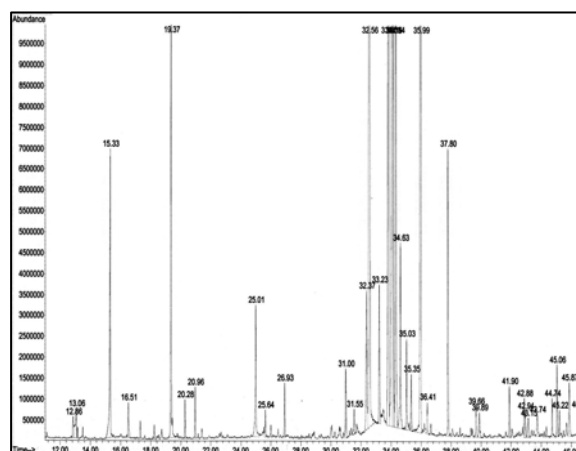


Fig. 3: Total Ion Chromatogram (TIC) of MSTFA derivatized methanolic fraction of ginger (*Z. officinale* Roscoe) leaves of Bukit Tinggi cultivar employing GC-MS.

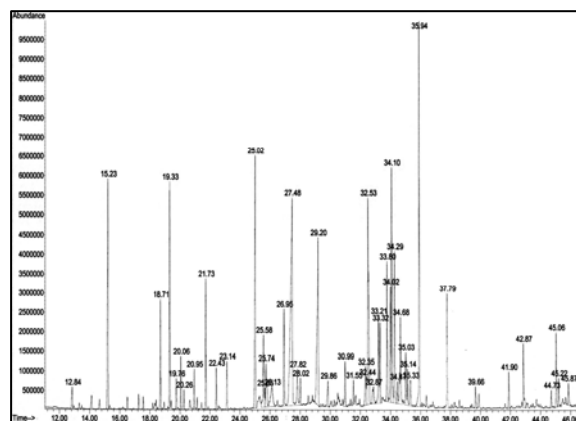


Fig. 4: Total Ion Chromatogram (TIC) of MSTFA derivatized methanolic fraction of ginger (*Z. officinale* Roscoe) leaves of Tanjung Sepat cultivar employing GC-MS.

In the other hand, a slightly difference in the level of metabolites were detected among the three ginger cultivars (Table 4 and 5).

Table 2: A qualitative comparison of the type of ginger metabolites in the methanolic fraction

Rt. \pm SD (n = 3) (min)	Identity	Cultivars			(m/z)
		BT	TS	SB	
19.70 \pm 0.04	L-Threonine	-	+	+	248
20.00 \pm 0.05	Glycine	-	+	+	276
21.68 \pm 0.05	L-Serine	-	+	+	278
22.38 \pm 0.05	L-Threonine	-	+	+	291
25.69 \pm 0.05	L-Aspartic acid	-	+	+	232
27.98 \pm 0.08	Glutamic acid	-	+	+	363
29.21 \pm 0.00	L-Asparagine	-	+	-	231
31.66 \pm 0.04	Ribonic acid	-	+	+	333
32.58 \pm 0.02	Sorbose	+	+	-	437

Note: BT: Bukit Tinggi; TS: Tanjung Sepat; SB: Sabah; Rt: Retention time; (+): Refer to present of compounds; (-): Refer to absence of compounds

Table 3: A qualitative comparison of the level of ginger metabolites in the chloroform fraction

Rt. (min)	Identity	Cultivars			(m/z)
		BT	TS	SB	
19.12	2-tertiobutyl cyclohexyl acetate	-	+	+	138
27.05	3,5-Dimethyl-2,4,6-tri chloro phenol	+	-	-	224
28.47	Carbamothioic acid	+	-	-	151
29.81	1,3,5-tricyano-2- (trichloro ethenyl) benzene	+	-	-	281
30.27	Thymine	+	-	-	270
32.58	Dibutyl phthalate	-	+	-	223
34.07	Normethandrone	+	-	-	288
35.14	2(1H)-Naphthalenone	-	+	-	208
37.85	Heptadecanoic acid	+	-	-	342
38.92	11-Tricosene	+	+	-	111
42.45	Ricinelaicid acid	+	-	-	328
48.98	Stigmasterol	-	-	+	484
49.66	Pentacosanoic acid	+	+	-	396
52.45	Cerotic acid	+	-	-	410
53.24	β -Sitosterol	+	-	+	396

Note: BT: Bukit Tinggi; TS: Tanjung Sepat; SB: Sabah; Rt: Retention time; (+): Refer to present of compounds; (-): Refer to absence of compounds

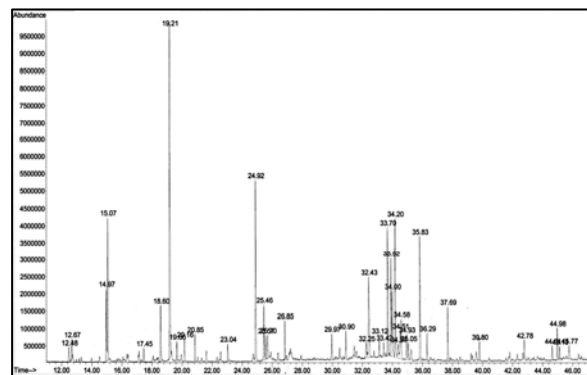
Fig. 5: Total Ion Chromatogram (TIC) of MSTFA derivatized methanolic fraction of ginger (*Z. officinale* Roscoe) leaves of Sabah cultivar employing GC-MS

Table 4: A quantitative comparison of the level of ginger metabolites in the methanolic fraction

Rt. \pm Sd (n = 3) (min)	Identity	Cultivars			(m/z)
		BT	TS	SB	
24.99 \pm 0.04	Malic acid	2	2	2	335
25.53 \pm 0.04	L-proline	1	1	2	258
33.19 \pm 0.04	β -D-galacto furanose	2	2	1	319
33.78 \pm 0.05	D-fructose	2	2	2	307
34.09 \pm 0.06	L-glucose 5 TMS	2	2	2	231
34.58 \pm 0.05	Glucose, oxime	1	1	1	319
35.31 \pm 0.04	α -D-glucopyranose	1	1	1	204
35.92 \pm 0.06	D-glucose	2	2	2	231
37.76 \pm 0.05	Myo-inositol	1	2	2	343
39.63 \pm 0.04	Glucose	1	1	1	231
41.87 \pm 0.04	D-manno-pyranose	1	1	1	219

Note: Rt: Retention time; BT: Bukit Tinggi; TS: Tanjung Sepat; SB: Sabah; (1): Indicates <0.5%; (2): Indicates 0.5-5%; of total integrated peak area of TIC of a particular sample

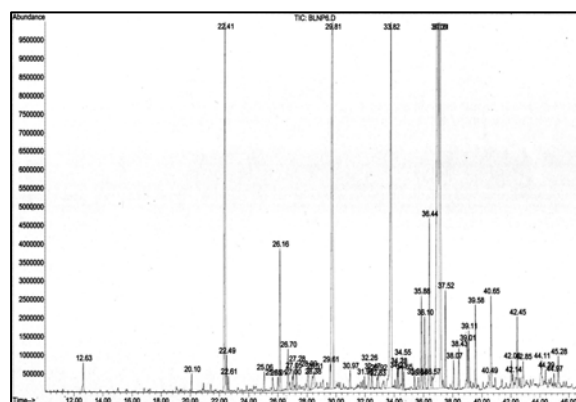
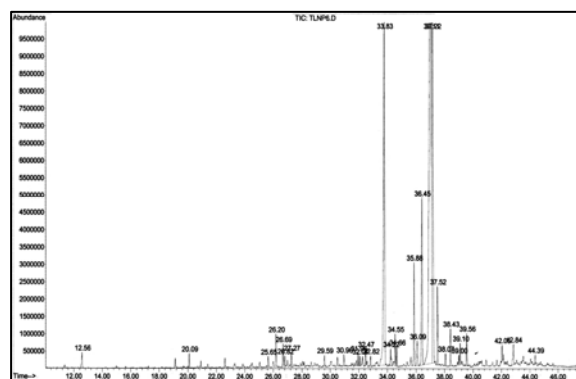
Fig. 6: Total Ion Chromatogram (TIC) of MSTFA derivatized chloroform fraction of ginger (*Z. officinale* Roscoe) leaves of Bukit Tinggi cultivar employing GC-MSFig. 7: Total Ion Chromatogram (TIC) of MSTFA derivatized chloroform fraction of ginger (*Z. officinale* Roscoe) leaves of Tanjung Sepat cultivar employing GC-MS

Table 5: A quantitative comparison of the level of ginger metabolites in chloroform fraction

Rt. \pm Sd (n = 3) (min)	Identity	Cultivars			(m/z)
		BT	TS	SB	
15.01 \pm 0.011	Meta-cresol	1	1	1	180
23.25 \pm 0.011	α -Neoclovene	1	1	1	204
25.05 \pm 0.000	Lauric acid	1	1	1	214
27.59 \pm 0.000	Tau-Cadinol	1	1	1	204
28.06 \pm 0.006	α -Gurjunene	1	1	1	204
28.66 \pm 0.004	(Z) -3-(4-n-Butyl-3-thienyl) propenal	1	1	1	194
29.09 \pm 0.013	m-Xylene	1	1	1	297
29.60 \pm 0.009	Myristic acid	1	1	1	242
30.10 \pm 0.027	α -Hexylcinnamic aldehyde	1	1	1	216
31.70 \pm 0.004	Pentadecanoic acid	1	1	1	256
31.96 \pm 0.004	Neophytadiene	1	1	1	137
32.25 \pm 0.004	Tetradecanoic acid	1	1	1	285
32.47 \pm 0.009	Neophytadiene	1	1	1	278
33.19 \pm 0.007	9-Hexadecenoic acid	1	1	1	236
33.82 \pm 0.011	Palmitic acid	3	3	3	270
34.28 \pm 0.004	Cinnamic acid	1	1	1	265
35.64 \pm 0.004	Margaric acid	1	1	1	284
36.09 \pm 0.007	Palmitic acid, TMS	2	2	2	328
37.08 \pm 0.024	Linoleic acid	3	3	3	294
37.20 \pm 0.022	Linolenic acid	3	3	3	236
37.51 \pm 0.013	Stearic acid	2	2	2	298
39.00 \pm 0.007	Linoleic acid, TMS	2	1	1	352
39.10 \pm 0.007	Oleic acid	2	2	2	354
39.56 \pm 0.004	Stearic acid, TMS	2	2	2	356
44.10 \pm 0.007	Behenic acid	2	1	1	354
45.64 \pm 0.004	Tricosanoic acid	1	1	1	368
47.28 \pm 0.004	Cholest-7-ene	1	1	1	399
47.43 \pm 0.000	Tetracosanoic acid	1	1	1	382

Note: Rt: retention time; BT: Bukit Tinggi; TS: Tanjung Sepat; SB: Sabah; (1) Indicates <0.5%; (2) Indicates 0.5-5%; (3) Indicates >5% of total integrated peak area of TIC of a particular sample

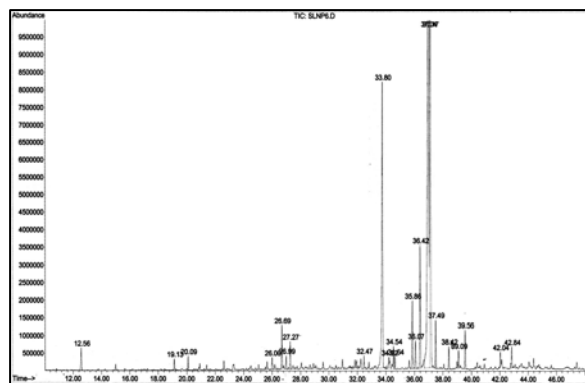


Fig. 8: Total Ion Chromatogram (TIC) of MSTFA derivatized chloroform fraction of ginger (*Z. officinale* Roscoe) leaves of Sabah cultivar employing GC-MS

DISCUSSION

The low percent of identified peaks (24-30%) both in methanolic and chloroform fractions of the three ginger cultivars. It might be due to some reasons such as peak overlapping, a low level of analytes (~0.05-1%) or simply, because no information is available so far in the spectra data base library.

A qualitative variation on the type of ginger metabolites was observed where some of metabolites were present in one but not in the other cultivars. In the other hand, a slightly difference in the level of metabolites were detected among the three ginger cultivars. It seems that these chemical variations have not caused by the geographical or environmental differences as micro-propagated ginger explants were used in this study. It could be due the genetic variations of the gingers. These results could not be attributed to the environmental factors since all these plants were grown under identical conditions, same composition of the culture media as well as same plants age.

Genes are differentially expressed under different environmental and intrinsic conditions such as nutrient availability (Oh *et al.*, 2002). Variation in gene expression rate will lead to variation in metabolites concentration. Accordingly, when we eliminate all effective external factors we expect to get the same level of gene expression and consequently no quantitative differences will detect and the metabolites concentration do not show any significant variation among the cultivars.

CONCLUSION

The GC-MS metabolic fingerprinting analysis of derivatized ginger leaves extract reveals the presence of chemical variation among the three ginger cultivars. Bukit Tinggi ginger cultivar showed the highest number of detected metabolites. In the other hand, Tanjung Sepat and Sabah cultivars showed almost the same number of detected metabolites. No marked differences in the concentrations of detected compounds among the three ginger cultivars leaf samples. Some of the detected metabolites can be used as biochemical markers for the identification and differentiation between ginger cultivars. These results were at the level of metabolic fingerprinting. A more precise method for detection of components concentration like metabolic profiling of specific metabolites group or target metabolic analysis may be needed to confirm the results.

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